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Retrospective Assessment of Radiation Exposure Using Biological Dosimetry: Chromosome Painting, Electron Paramagnetic Resonance and the Glycophorin A Mutation Assay

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Biological monitoring of dose can contribute important, independent estimates of cumulative radiation exposure in epidemiological studies, especially in studies in which the physical dosimetry is lacking. Three biodosimeters that have been used in epidemiological studies to estimate past radiation exposure from external sources will be highlighted: chromosome painting or FISH (fluorescence *in situ* hybridization), the glycophorin A somatic mutation assay (GPA), and electron paramagnetic resonance (EPR) with teeth. All three biodosimeters have been applied to A-bomb survivors, Chernobyl clean-up workers, and radiation workers. Each biodosimeter has unique advantages and limitations depending upon the level and type of radiation exposure. Chromosome painting has been the most widely applied biodosimeter in epidemiological studies of past radiation exposure, and results of these studies provide evidence that dose-related translocations persist for decades. EPR tooth dosimetry has been used to validate dose models of acute and chronic radiation exposure, although the present requirement of extracted teeth has been a disadvantage. GPA has been correlated with physically based radiation dose after high-dose, acute exposures but not after low-dose, chronic exposures. Interindividual variability appears to be a limitation for both chromosome painting and GPA. Both of these techniques can be used to estimate the level of past radiation exposure to a population, whereas EPR can provide individual dose estimates of past exposure. This paper will review each of these three biodosimeters and compare their application in selected epidemiological studies. © 2006 by Radiation Research Society

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INTRODUCTION

Biological monitoring of dose can contribute important, independent estimates of cumulative radiation exposure in epidemiological studies for individuals and population groups, especially in studies in which physical dosimetry is lacking. Biological markers of exposure can be used to validate physical measurements and model-based dosimetry or to characterize the level of exposure in the absence of individual dosimeters (physical dosimetry). The three biological markers that will be reviewed in this paper are fluorescence *in situ* hybridization (FISH) for chromosome translocation analysis of peripheral blood lymphocytes or chromosome painting, glycophorin A somatic mutation assay (GPA) of red blood cells, and electron paramagnetic resonance (EPR) of tooth enamel. These three biomarkers of exposure were selected because they have been applied most widely in epidemiological studies of past radiation exposure. The choice of which biological dosimeter to apply depends upon the level of exposure (high-dose or low-dose), mode of exposure (acute or chronic), time since exposure (recent or past), type of radiation (e.g. X rays, γ rays, neutrons), sensitivity and specificity of the assay, laboratory requirements, and availability of blood or teeth.

Chromosome painting for translocation analysis is a somatic mutation assay that measures the frequency of chromosome aberrations in white blood cells, whereas GPA is a somatic mutation assay that measures the frequency of mutations in red blood cells. EPR detects the presence and concentration of free radicals in calcified tissue of teeth. All three methods are described and compared in terms of the basic methodology, validity, advantages and limitations, practical considerations and future direction, with a particular emphasis on their application in epidemiological studies of populations exposed primarily to photons (Table 1).

CHROMOSOME PAINTING

Introduction

Chromosome alterations have been known to be associated with cancer for many decades (*1*). Specific chromo-

TABLE 1
Selected Biological Dosimeters Used for Retrospective Dose Assessment in Irradiated Populations

Characteristic	Method		
	Chromosome painting	Electron paramagnetic resonance (EPR)	Glycophorin A somatic mutation assay (GPA)
Technique			
Source material	Blood, lymphocytes	Teeth, enamel	Blood, erythrocytes
Culture conditions	Cells need to be prepared 48–72 h within blood drawing	At room temperature indefinitely—separate enamel from dentin	Stored at refrigerator temperature up to 2 weeks prior to analysis
Analytical method	Fluorescence *in situ* hybridization (FISH)	EPR spectrometer	Flow cytometry
Outcome	Translocations and dicentrics (stable and unstable chromosome aberrations)	Radiation-induced free radical signals	Variant frequency of GPA alleles (NØ, MØ, NN, MM)
Radiation exposure			
Radiation type	γ rays, X rays, neutrons	γ rays, X rays, β particles	γ rays
Minimum detectable dose	~10–20 cGy	30 mGy	10–20 cGy most useful at ≥1 Gy
Time limitation	Up to several decades after exposure, cumulative	Up to several decades after exposure, cumulative	Up to several decades after exposure, cumulative
Individual dose assessment	Yes, but interindividual variation may be high	Yes	Yes, but interindividual variation is high
Epidemiologic considerations			
Modifier of dose response	Age and tobacco smoke	UV-radiation exposure	None
Application to irradiated populations (whole-body exposures)	A-bomb survivors, accident victims, Chernobyl cleanup workers, radiation workers, residents near nuclear test sites and nuclear facilities	A-bomb survivors, radiation workers, Chernobyl cleanup workers, residents near nuclear test sites and nuclear facilities	A-bomb survivors, radiation workers, Chernobyl cleanup workers, hospital workers
Advantages	Well-characterized dose–response curves	Individual dose assessment, low minimum detectable dose	Practical to use in field conditions, small amount of blood required
Disadvantages	Interindividual variability affects usefulness as an individual biodosimeter	Requires teeth, cannot separate UV from ionizing radiation contribution	Only 50% of the population is eligible for assay; no *in vitro* assay
Practical considerations	Reliable, speed of analysis, but expensive	Reliable, but expensive	Not useful for exposures <1 Gy; inexpensive

some rearrangements can be diagnostic of tumors, and some aberrations are thought to play a causative role in tumorigenesis, growth and metastasis (*2*). The well-known relationships between radiation, chromosome damage and cancer, in addition to the existence of well-characterized radiation dose–response curves, makes the analysis of chromosome aberrations for assessments of external radiation exposure highly relevant.

Structural chromosome aberrations arise from DNA double-strand breaks that are misrepaired or not repaired. Double-strand breaks may also be repaired correctly, at least when viewed at the cytological level, but such repair may

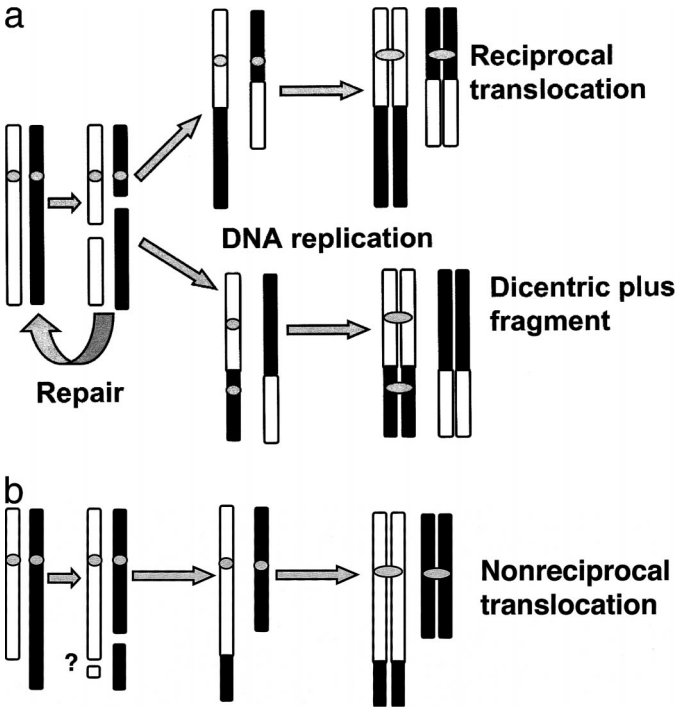

FIG. 1. Schematic showing how translocations and dicentrics form. Panel A: Dicentric and reciprocal translocation. Panel B: Nonreciprocal translocation.

in fact contain submicroscopic alterations in the DNA sequence that are detrimental to the affected cell (*3*). Figure 1a shows several types of chromosome exchanges that are the most relevant to biological dosimetry. These are the translocation and the dicentric, sometimes referred to as symmetrical and asymmetrical chromosome exchanges, respectively. Translocations are classically viewed as representing an exchange of material between two chromosomes such that each derivative chromosome has a single centromere, which is the point of attachment for spindle fibers during mitosis. The alternative exchange pattern yields a chromosome with two centromeres, called a dicentric, and an acentric fragment. Translocations and dicentrics theoretically are induced at equal frequencies.

In actual practice, the types of rearrangements formed after radiation exposure are more complicated because not all translocations appear reciprocal (Fig. 1a). Nonreciprocal translocations (Fig. 1b), also known as one-way translocations, stand in contrast to reciprocal translocations, which are also known as two-way translocations. Nonreciprocal translocations involve the transfer of material from one chromosome to another without any apparent reciprocity, and they are problematic for several reasons. First, the absence of a cytologically apparent exchange between two chromosomes does not necessarily mean that no such exchange has occurred. Rather, the amount of material translocated onto one of the chromosomes may simply be below the limit of cytological resolution (*4, 5*). Second, an apparent nonreciprocal exchange may be the result of a multiway exchange involving more than two chromosomes, which is only partially visualized by the chromosome painting probes used (*6–8*).

This paper focuses primarily on translocations because they survive cell division and thus are the aberrations of choice for retrospective biodosimetry. Some discussion of dicentrics is also provided for sake of comparison.

Fundamentals

Fluorescence *in situ* hybridization (FISH) with whole-chromosome paints has gained wide popularity in recent years for assessing chromosome damage. Painting involves hybridization of the probe, detection of the hybridized probe, and counterstaining of the slide to visualize the unpainted chromosomes. Successful painting produces chromosomes that are uniformly and brightly labeled along their entire length (Fig. 2). When whole-chromosome painting first became available (*9, 10*), its potential applications to biological radiation dosimetry were obvious. However, evaluation was necessary to determine whether painting gave estimates of chromosome aberration frequencies similar to those determined with other methods in use at the time. This included conventionally stained (''unbanded'') chromosomes as the predominant method as well as the occasional use of banded chromosomes. Replicate slides from cultures of human whole blood that had been irradiated with ^{137}Cs γ rays were used, and results obtained by painting compared with those obtained from unbanded and banded chromosomes yielded frequencies of dicentrics that were equivalent to each other at all doses examined (0.23–4.0 Gy) (*11, 12*). Translocation frequencies were also shown to be equivalent using different methods.

Two outcomes of the validation process surprised some investigators. Frequencies of translocations and dicentrics were not always equivalent, as had been expected. One possible explanation involved mis-scoring of exchanges (*13*), while other evidence supported the hypothesis that some types of translocations were detected more readily than others (*11*). Underlying the problem of this apparent discrepancy was the knowledge that chromosome denaturation, which is essential to successful DNA hybridizations, diminished chromosome morphology and in some cases made centromeres difficult to identify. Some investigators responded by fluorescently labeling centromeric regions simultaneously with whole-chromosome painting probes, e.g. (*5, 14*), whereas other investigators relied on counterstains such as DAPI and classical analysis techniques to identify centromeres (*15–18*). Each approach has advantages and disadvantages, and both are valid and enjoy widespread use.

The second unexpected outcome concerned the presence of cells with complex rearrangements, defined as three or more breaks in two or more chromosomes (*19*). Complex aberrations are now known to be prevalent in cells acutely exposed to doses above 2 Gy (*11, 20*). However, complex

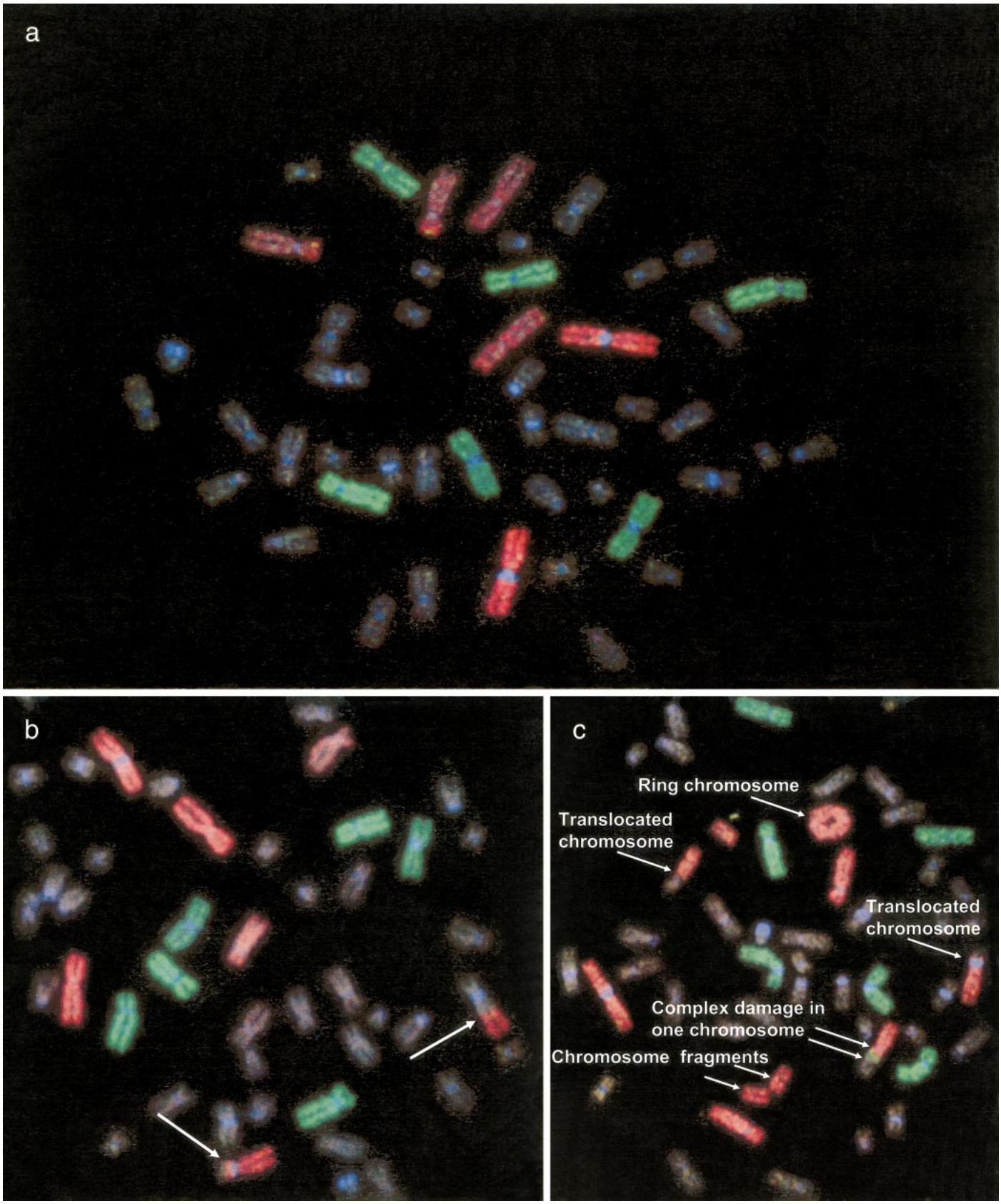

FIG. 2. Human peripheral blood lymphocytes showing chromosomes 1, 2 and 4 painted red (22.07% of the genome) and chromosomes 3, 5 and 6 painted green (18.02% of the genome). This painting scheme detects 56% of all simple exchanges [(*37*) and Eq. (A5) in text]. Panel A: Normal cell. Panel B: Cell with a reciprocal translocation. Panel C: Complex cell with many types of aberrations.

rearrangements have been observed at low doses and even in cells with no apparent radiation exposure. Of relevance to epidemiological studies is the possibility that complex aberrations may arise from exposures other than ionizing radiation, such as cigarette smoking. Cells with complex damage are not necessarily the same as rogue cells, which have very large numbers of aberrations (*21–24*). For dosimetry, the most significant problem concerning complex damage concerns the manner in which these events should be used to estimate doses. Prior to painting, dosimetry was usually based on the frequencies of dicentrics, which were generally assumed to involve breaks and exchanges between exactly two chromosomes. Similar assumptions were made concerning translocations. This view has yielded to the concept that multi-way breaks and exchanges not only occur but are also common after acute high doses (*6–8*).

Most applications of chromosome painting for dosimetry have involved labeling a few chromosomes in one or two colors, with the remaining chromosomes counterstained in a different color. When only a few chromosomes are painted, it is possible that complex aberrations may not be fully identified (*6*). Multicolor painting has now clearly demonstrated that some ambiguity in identification of aberrations will always be present unless every chromosome is painted in a unique color (*7*). Thus, while the problem of complex aberrations as applied to dosimetry is real, there appears to be no pressing demand to reach a widely accepted solution.

Persistence of Aberrations

Frequencies of some types of aberrations such as dicentrics decline with time after exposure (*25, 26*). Among cells with a dicentric chromosome, half will die during each cell division. This contrasts with translocations in which each derivative chromosome has a single centromere, allowing unencumbered transmission to the daughter cells. Until recently, the frequencies of translocations were generally assumed to remain unchanged after exposure. However, translocation frequencies decline after exposure, as shown in numerous studies including work performed *in vitro* (*20, 27*) in animals (*28–30*) and humans (*15, 31–35*). Unlike dicentrics, translocation frequencies do not decline to near-zero values. Instead they reach a non-zero asymptote, which appears to be dependent on dose, enabling translocations to retain their ability to be used for dosimetry many years after exposure. However, the kinetics of translocation loss in humans has not been well characterized, because there have been relatively few opportunities to perform such studies. The accident at Goiania is a notable and important exception (*32*). Finally, studies of the persistence of different types of translocations have shown that reciprocal translocations have a greater probability of surviving cell division than do nonreciprocal translocations, e.g. (*27, 30, 35*), because some nonreciprocal translocations are part of a complex aberration, which has a greater probability of being lethal.

For biological dosimetry, especially when years have elapsed since exposure, it is critically important to keep in mind the differential persistence of translocations. This may have a direct bearing on the ability to reconstruct doses. For this reason, it has been suggested that only reciprocal translocations be used for dose reconstruction (*36*). Although this approach appears to avoid the problem of differential persistence, it ignores the existence and importance of complex rearrangements and could mean discarding large amounts of data that may be relevant. Perhaps the best approach is one that uses all the translocations observed to estimate dose. This means recording all the different types of aberrations observed in every cell, which preserves the ability to go back to the original observations and reanalyze the data. To facilitate this, it is recommended that photographs (with either a digital or 35-mm camera) of all abnormal cells be made and archived since fluorescence signals can fade with time. This allows the investigation of questions that were not anticipated in the original study design (*37*).

Conversion of Metaphases to Whole-Genome ''Cell Equivalents''

Chromosome painting identifies only a portion of all the possible exchanges within a given cell. Identification of aberrations is based on the ability to see color ''junctions'', i.e. locations along a chromosome where one color ends and another begins. For this reason, exchanges that occur between chromosomes labeled in the same color will not be observed. The sizes of the chromosomes (*38*) painted in each color will directly determine the proportion of the genome painted, which in turn determines the fraction of all exchanges observed. See Appendix 1 for conversion of metaphases to whole-genome ''cell equivalents''.

Choice of Chromosomes to Paint

The primary consideration for most biodosimetry applications using cytogenetics is the detection of aberrations in as much of the genome as possible. This means painting as many chromosomes in as many colors as feasible. As noted above, the more chromosomes that are painted, the greater the certainty of identifying the aberrations completely. This is especially important if complex exchanges are of concern, such as in studies involving high-linear energy transfer (LET) radiation, or where high doses of low-LET radiation may be encountered. For low doses of low-LET radiation, or for chronic exposures of low-LET radiation even when the total dose may be high, it might be sufficient to paint a few chromosomes in a single color. In general, painting a few large chromosomes rather than many small chromosomes is the choice of most investigators because this simplifies hybridizations and microscopic analyses.

Chromosomes differ in their radiation sensitivity [see (*39*) and references therein], and certain regions of chromosomes may be prone to chromosome exchanges (*37, 40*).

Although the physical and molecular basis of non-random chromosome breakage and repair are not well understood, investigators would do well to keep such non-random effects in mind, particularly if only a few chromosomes will be painted. Making sense of the vast array of aberrations necessarily requires some process of simplification. Soon after painting became routine, two complementary nomenclature systems were developed to handle this complexity. These are known as the S&S system (*19, 41*) and PAINT (Protocol for Aberration Identification Nomenclature Terminology) (*6*), and a direct comparison of these systems has been published (*42*).

Modifiers of Response

When chromosome translocations are used as an end point in epidemiological studies, several variables must be considered during the design and the analysis phases. The most common of these is subject's age and smoking status, e.g. (*18, 34, 43–45*), because both have been significantly associated with increases in translocation frequencies in almost every study with sufficient statistical power. Translocations can be viewed as a dosimeter that is the integral of all of life's exposures (e.g. chemical, viral, environmental), including but not limited to radiation. For example, translocations accumulate with age, because of the stability of these rearrangements through cell division. Exposures besides those directly under investigation must also be carefully assessed, including other sources of radiation and chemical exposure. The influence of factors other than environmental exposures should also be considered. The ''rapid acetylase'' genotype at the *N*-acetyltransferase 2 locus has been reported to be associated with increased translocation frequencies in cigarette smokers aged 60 and older (*46*). This illustrates that the effects of environmental exposure, genotype and age on translocation frequencies are not always obvious *a priori* and can be complicated. Differences in interindividual susceptibility are likely to gain importance as our understanding of human exposure responses increases. The phenotype of genetic susceptibility has been observed in individuals identifiable as mutagen sensitive on the basis of an *in vitro* challenge assay, which measures the number of bleomycin-induced chromatid breaks in short-term culture (*47, 48*). Mutagen-sensitive individuals number approximately 25% of the population (*47, 49*). They have no obvious genetic disorders, but they are thought to have subtle differences in DNA repair capacity (*49, 50*) even though the molecular basis of this phenotype has not been determined. Individual mutagen sensitivity status has been shown to be constant over time and, in healthy subjects, independent of cigarette smoking (*51–53*) and other environmental influences (*52–55*).

Clones

Clones (i.e., the same set of rearranged chromosomes found to occur in at least three cells) have been observed in subjects exposed to radiation as well as those with no known history of exposure (*31, 39, 56–58*). Clones have been detected as well in mice acutely exposed to radiation and followed throughout their life span (*30*). It should be noted that clones may arise *in vivo* either before or after exposure. Screening for clones should be limited to those abnormal cells that have aberrations capable of surviving mitosis; all other cells can be ignored. Clones are most easily identified by examining photographs, which is one reason why it is important to archive images of all abnormal cells as noted earlier. It is also possible to make statistical estimates of which subjects have clones provided a record is made of the chromosomes involved in every translocation or insertion (*39*). Once clones have been identified, the manner in which the data are handled will depend on the question being asked. For individual dosimetry calculations, counting all clonal cells as a single mutational event is the method of choice (Appendix 2). However, there are good reasons for making no adjustment at all, because clones of normal cells also exist. Thus, on average across an entire population, the mean frequency of translocations per cell should not depend on the size or presence of clones of either normal or abnormal cells, presuming that there is no selective advantage or disadvantage to cells with or without translocations.

Microscope Slide Scoring and Quality Control

Given the large size of many epidemiological studies, it is unreasonable to expect that a single individual will perform all the microscope analyses. This introduces the need for consistency in identification and enumeration of aberrations among multiple slide readers. Extensive training of readers is recommended in addition to photographing all abnormal cells for subsequent confirmation by a second highly trained observer. All slides are coded to prevent observer bias. To ensure that the readers are scoring the aberrations correctly, replicate slides from each research subject should be read by at least two different readers. Periodically, slides from control subjects that contain cells that were irradiated *in vitro* with a known dose should be read by each reader to determine their accuracy.

Standard Values from Reference ''Control'' Populations

Large population studies typically have a well-matched reference ''control'' population. However, for studies of one or only a few exposed individuals, comparison to a correspondingly small control population may not be satisfactory, given the importance of variables such as age and smoking. Therefore, it is helpful to have available historical data against which appropriate comparisons may be made, even if these data are not from the same laboratory. Data that may be appropriate for this purpose exist in the peer-reviewed literature but are not readily accessible in a form that could be used. Several years ago, historical data at the Lawrence Livermore National Laboratory were used ex-

actly for this purpose when evaluating the exposure history for one subject (*59*). The lowest dose detectable with translocation analysis depends on many factors, including the baseline frequency of translocations in the exposed subject(s), the dose rate and perhaps the type of radiation received.

Use of FISH in Epidemiological Studies after Whole-Body Exposures

Whole-chromosome painting for radiation biological dosimetry has been applied to many exposed populations. Among these are the A-bomb survivors (*60*), Chernobyl (*18, 45, 61–64*), Sellafield British Nuclear Fuels workers (*43, 65*), populations in Mayak/Techa River (*14, 66–68*), Semipalatinsk (*17, 69*), Taiwan (*70*), Estonia (*16, 45*), and Goiania (*32, 45*), as well as various occupationally exposed groups (*71*). It is beyond the scope of this paper to discuss all of these studies in detail. However, several are worth highlighting. In the Sellafield workers, it was shown that chronic exposures produced approximately sixfold fewer chromosome aberrations per unit dose compared to the acute exposures received by the A-bomb survivors. These results not only provide solid evidence for the accumulation of translocations under conditions of chronic occupational exposure, but they also indicate that translocations persist for decades. In a study of thyroid nodularity and cancer among Chernobyl workers from Estonia (*72*), nodularity showed a nonsignificant positive association with the proportion of lymphocytes with chromosome translocations. The mean documented population dose was 10.8 cGy, which was substantially lower than expected. This result was subsequently confirmed in an independent study (*18*), which demonstrated the ability to detect a significant increase in translocations as a result of radiation exposure in the presence of two modifying factors, aging and cigarette smoking. Providing dosimetry for such low doses is possible on a population basis, as many of these studies show, but it may not be possible to obtain data with sufficient accuracy for individual subjects. The most notable feature in common among these studies is that all involved retrospective biological dosimetry, which was made possible, or significantly enhanced, by the analysis of translocations identified by chromosome painting. These papers as well as many others reporting on the use of FISH for translocation analyses have made major contributions to our understanding of the long-term risks of exposure to ionizing radiation.

Advantages

When performing biological dosimetry using cytogenetic techniques a long time after exposure, it is necessary to estimate the increase in translocation frequencies associated with aging while simultaneously determining the decrease in translocation frequencies that occurs after exposure. This problem, as well as the expansion of clones of abnormal cells, has been specifically addressed using a mouse model (*30*). This work demonstrates the feasibility of performing meaningful biodosimetry, even many years after exposure, while accounting for the presence of multiple confounding factors.

Chromosome painting has one critical advantage compared to classical cytogenetic methods, namely speed of analysis. In our experience (JDT), people trained in the identification of aberrations and in fluorescence microscopy routinely score an average of 250 metaphase cells per hour, which is the same as 140 whole-genome equivalents (Appendix 1) with the painting scheme shown in Fig. 2. Compared to Giemsa-banded cells, which can be scored at a rate of approximately four cells per hour, this is a 35-fold increase in throughput. This translates into significant savings in labor costs, leading to improved statistics and better dosimetry. Increasing the number of cells scored also permits detection of aberrations at lower doses and improves the likelihood of performing meaningful biodosimetry at low doses. Another important advantage is the inherently objective nature of the assay. Aberrations are identified on the basis of color junctions for chromosome exchanges (Fig. 2) or additional color signals for acentric fragments (or aneuploidy), both of which are easy to see and unlikely to be overlooked in the analyses.

Limitations

The primary limitation of chromosome painting is cost. Probes are expensive and analysis requires expensive microscopes to visualize the fluorescence signals. Most laboratories paint a small number of chromosomes in one or a few colors. Therefore, not every set of chromosome exchanges will be understood in its entirety, and certain assumptions will be needed when counting aberrations for dose assessment. In many epidemiological studies of radiation exposure, doses are low and/or received over a long time, the majority of aberrations observed are simple (*73*), and dosimetry is straightforward. The problem of fully identifying complex aberrations is confined primarily to acute doses above $\sim$2 Gy, which normally comprise only a small portion of exposures.

Other limitations of chromosome painting include missing some aberrations if exchanges between chromosomes are labeled in the same color. Intrachromosomal exchanges such as inversions will not be seen unless they cause obvious changes in the chromosome arm lengths, but since these rearrangements do not produce color junctions, painting will not aid in their detection. Furthermore, small amounts of exchanged material cannot always be seen, although the level of resolution is certainly better than with banding. Some types of translocations will be more visible than others. For example, a small amount of chromosomal material that is painted red and translocated onto a counterstained blue chromosome will be easier to see than the

reverse, a problem that has been addressed previously (*11, 74*).

Future Directions and Innovations

Overall, the advantages of chromosome painting outweigh its limitations, and painting is likely to remain one of the methods of choice for addressing most radiation biodosimetry needs for populations for some time to come. Establishment of a set of baseline translocation frequencies from hundreds or thousands of individuals representative of the wide social, cultural, ethnic and age distributions of our society should be a priority. Systems that use early biochemical responses to radiation, such as changes in gene expression, warrant serious research efforts. While some preliminary data are encouraging (*75*), novel approaches in cytogenetics are required to meet the challenges of the future.

EPR BIODOSIMETRY WITH TEETH

Introduction

Estimation of cumulative radiation exposure on an individual level is desirable, but it is often difficult to achieve in epidemiological studies of irradiated populations. EPR offers a method of individual retrospective dosimetry by measuring a radiation-induced free radical signal in tooth enamel (*76–84*). EPR, which was first applied as a retrospective epidemiological dosimeter to A-bomb survivors, revealed a good correlation with estimates of external whole-body dose (*76*). Since then, EPR has been used in several other irradiated populations in which it was possible to collect teeth from study participants.

Fundamentals

Tooth enamel, which is composed mainly of hydroxyapatite (96%), has a high sensitivity to ionizing radiation, which makes it suitable for radiation dosimetry. Radiation dose information is stored in tooth enamel as radiation-induced radicals of CO_2^- (*85*) with long-term stability of 10^8–10^{11} years (*86*). Due to the absence of cell structure, tooth enamel has extremely low metabolism and a stable mineral composition. Once enamel has been formed and has matured, it can be changed only by chemical and physical action of its environment, but it cannot self-repair or regenerate. Other significant features of tooth enamel as a dosimeter include a linear dose dependence of the concentration of radiation-induced radicals up to 300 Gy, low radiation dose detection limit (<100 mGy), and sensitivity to γ radiation, β-particle radiation and X rays.

EPR consists of resonant absorption of microwave energy by electron magnetic moments (or spins) in the sample under investigation. Resonance is the preferential absorption at a specific applied magnetic field, which is determined by the difference between energies of the electron magnetic moments. As a result of the microwave energy absorption, the number of electrons with magnetic moments opposing the magnetic-field direction is increased, and the number of the electrons with magnetic moments parallel to magnetic field is decreased. As soon as the sample under investigation exits the resonance condition, the original numbers of electrons with magnetic moments, parallel and opposed to the magnetic field, are restored. This implies that EPR is nondestructive and the measurement can be repeated an unlimited number of times with the same (within error limits) result. The main source of the electron magnetic moments in tooth enamel is unpaired electrons created by exposure to ionizing radiation. There have been attempts to use thermoluminescence and optically stimulated luminescence for dose estimation (*87*) but with limited success. Currently, EPR remains the only feasible method for dosimetry with tooth enamel due to its high sensitivity to radiation-induced radicals, nondestructive readout, and the small sample mass (<100 mg) required for measurements.

Different microwave frequencies can be used for EPR measurements, but most commercial EPR spectrometers operate in X-band (9–10 GHz), because it provides a good compromise between sensitivity, sample size and water content effects. The EPR spectrum of irradiated tooth enamel consists of several signals that can be divided into two categories, radiation-induced and radiation-insensitive signals. This approach is an approximation because the intensity of the so-called radiation-insensitive component is also slightly affected by ionizing radiation; however, it is evident only after irradiation with doses above 100 Gy. These EPR spectral components can be considered as radiation-insensitive in the application range of retrospective dosimetry.

The majority of radiation-induced radicals in tooth enamel are derived from carbonate. Not all radiation-induced radicals are thermally stable, and unstable signals decay completely at room temperature during the first 2 weeks after irradiation (*85, 88*). After irradiation, the signal amplitude gradually increases, possibly due to contributions from unstable signals, and reaches (at room temperature) a steady state after about 2 weeks.[2] The signal increase can be fixed by annealing the tooth enamel sample after irradiation at 90°C for 2 h (*88*). The signal also displays a linear dose response, which allows reconstruction of radiation dose by simple linear back extrapolation (Fig. 3).

Outline of EPR Dose Reconstruction

EPR biodosimetry consists of several steps, e.g. sample collection, sample preparation, EPR measurements, dose calibration and determination of the accidental dose. Table

[2] For dose reconstruction, the peak-to-peak amplitude of asymmetric EPR signal with $g_1 = 2.0018$ and $g_2 = 1.9971$ (signal maximum at $g = 2.0032$ and minimum at $g = 1.9971$) is used (Fig. 3). The signal is derived predominantly from stable CO_2^- radicals. The g factor of the EPR signal is a fundamental characteristic of a specific type of radical (induced by radiation or oxidation), and it reflects the signal position on an EPR spectrum.

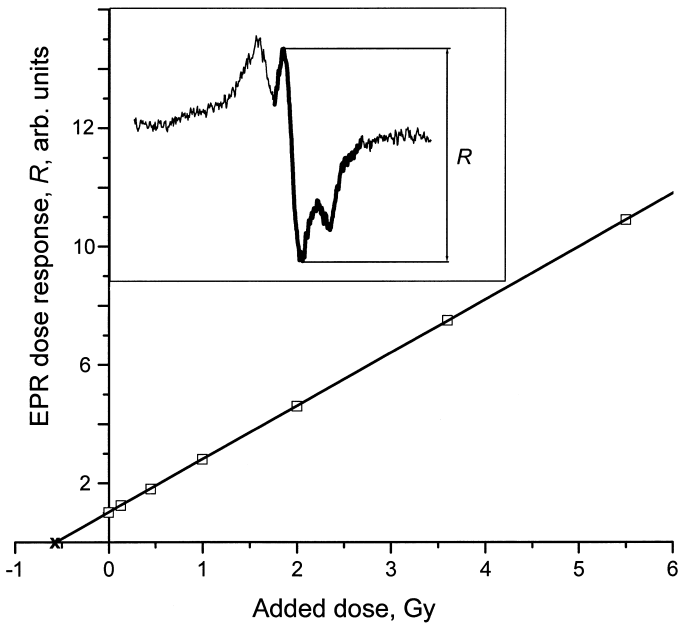

FIG. 3. Typical dependence of the EPR dose response, *R* on added dose. Open squares (□) show results of EPR measurements in original (nonirradiated) tooth enamel and at different added doses. Solid line is linear regression of the measurements. Cross (×) shows reconstructed radiation dose in the sample. The inset is an EPR spectrum of irradiated tooth enamel (0.6 Gy). Bold line shows radiation-sensitive part of the spectrum. *R* is a peak-to-peak amplitude, which is used for EPR dose reconstruction.

2 provides brief information on the specific aim of each step and some potential obstacles.

Sample collection. Human dentition consists of two generations, and both are suitable for EPR dose reconstruction purposes, although not equally. The first generation is known as the deciduous (often called milk or baby teeth) dentition and the second as the permanent (secondary) dentition. The infant jaw contains 20 deciduous teeth, which are much smaller in size than permanent teeth. Moreover, dose accumulation is limited by the relatively short period of childhood (age 1–12 years). Because of their small size, deciduous teeth have been measured mostly as a whole tooth including enamel and dentin (*89*, *90*). The lower limit of detection (LLD) for whole deciduous teeth has been estimated as 100 mGy (*90*). Permanent teeth are preferred in EPR retrospective dosimetry because they are bigger and accumulate radiation dose during almost the entire life of the donor. The large size of the permanent teeth also allows separation and purification of tooth enamel. This improves radiation sensitivity, and the LLD for purified tooth enamel prepared from a permanent molar was reported as 29 mGy (*91*). Human adults have 32 permanent teeth that erupt at different ages. For example, first molars erupt at about 6 years, second molars at 10–12 years, and third molars (wisdom teeth) between 18–30 years. Therefore, it is very important to obtain detailed information on the tooth position and birth year of the donor. These differences in eruption time between different teeth of the same individual can have an impact on dose reconstruction results (*92*). In addition, due to the substantial impact of sunlight on front teeth, tooth enamel from molars and premolars is the most suitable for EPR measurements (*93*).

TABLE 2
Main Steps of EPR Dose Reconstruction

Step	Task	Obstacles
Sample collection	Obtain teeth with tooth position, tooth donor age, residency and dosimetric information, which after EPR dose reconstruction measurements will give dose values received from the exposure event under study.	1. Teeth in different positions can have different time schedules of dose accumulation. 2. Front teeth may have measurable dose from sunlight exposure. 3. Some teeth could provide insufficient (<50 mg) tooth enamel because of caries or other dental diseases.
Sample preparation	Reduce or eliminate nondosimetric component of tooth enamel EPR spectrum, which obscures radiation response.	1. Presence of dentin having up to 30% of water and organics in its composition with strong nondosimetric signal. 2. Presence of the organic component in tooth enamel.
EPR measurements	Extract dose response from total EPR tooth enamel spectrum.	Possible spectrum distortion caused by inappropriate recording conditions.
Dose calibration	Determine measured EPR radiation response in terms of radiation dose absorbed in the specific organ of interest.	1. Energy dependence of EPR radiation response. 2. Possible existence of the dose distribution over the entire body.
Determination of the accidental dose	Evaluate other possible dose contributions besides accidental one, e.g. from radiation background and medical exposure.	Existence of several other dosimetric contributions in total lifetime accumulated dose measured by EPR.

The lack of a systematic approach to collecting teeth has characterized some previous work using EPR, and the following steps are suggested to improve standardization and the value of this method for estimating radiation dose.

1. Every tooth should be kept in a separate envelope with information on its position, tooth donor identification and age.
2. Teeth with steel or metal crowns or tooth roots (teeth with completely destroyed crowns) should not be collected, because they contain only small amounts of tooth enamel.
3. Front teeth are less suitable for EPR, because they might have a dose contribution from solar light.
4. Wisdom teeth should not be collected because they erupt much later than other teeth and will not reflect the dose contribution from radiation events that occurred before their eruption.

Sample preparation. For permanent molars, the procedure of sample preparation involves separation of the tooth enamel from dentin. This can be done mechanically with a dental drill or chemically. Chemical separation also purifies the sample, yielding improved dosimetric properties (*94, 95*), but care should be taken not to destroy radiation defects in the enamel by too vigorous treatment or too high temperature.

EPR measurements. An EPR spectrometer is a widely accepted tool used for investigating different types of free radicals. It allows broad variation in experimental conditions of spectra recording. However, not all combinations are suitable for recording tooth enamel spectra. For example, use of too high microwave power and/or amplitude of high-frequency modulation may cause spectrum distortion, which could result in nonlinearity of the EPR dose response. In addition, selection of values that are too low will result in noisy spectra, which could significantly increase the dose reconstruction error. Therefore, a proper balance between spectrum enhancement and possible distortion should be maintained. Recently the procedure of optimization of EPR spectrometer parameters was investigated by Ivannikov *et al.* (*96*). Another consideration for measurement of EPR dose response at low doses ($<$300 mGy) is interference from the radiation-insensitive component (Fig. 3). To extract the EPR dose response of tooth enamel at low doses, several different methods can be applied [see for review (*97*)]; however, at present, most researchers prefer to use computer modeling of EPR spectra [see for example ref. (*98*)].

Dose calibration. The measured EPR response of tooth enamel (peak-to-peak amplitude of the EPR signal, R in Fig. 3, or its corresponding parameter if computer modeling is applied) is converted into absorbed dose. Two methods have been used to determine the absorbed dose. They are additive reirradiation and the ''universal'' calibration curve. Additive reirradiation consists of incremental irradiation of the tooth enamel sample to construct a response curve specific to the sample being investigated. This method typically requires four to five additional dose increments. A study of the variation of dose coefficients in multiple samples found that EPR radiation sensitivity and variability depend mostly on the sample preparation procedure (*99*). The maximum variability in EPR sensitivity of enamel from Egyptian teeth, for example, was up to 10%, and the mean sensitivity was in good agreement with that of German teeth. Therefore, in most EPR dose reconstructions, the ''universal'' calibration curve method could be used. In this case, the procedure of dose calibration involves creation of a calibration curve using specially prepared samples. For example, this can be accomplished by mixing small portions of the samples under investigation. The parameters of the calibration line, slope and intercept with dose axis are determined by linear regression analysis. Benefits of the ''universal'' calibration method are that it is nondestructive and much less time-consuming. The 2nd International Dose Intercomparison demonstrated that the ''universal'' calibration method produces accurate dose reconstruction results (*100*).

Determination of the Accidental Dose

EPR measures the cumulative lifetime absorbed dose to the tooth of the donor, which consists of accidental or occupational exposure, as well as contributions from background and medical doses. Accidental doses are often comparable to or even less than the background component. For example, this was the case for the EPR dose reconstruction for the population exposed to the Chernobyl accident (*93, 101, 102*). To determine the contribution of background radiation, teeth from a control or ''unexposed'' group that had similar lifestyles and work conditions as the exposed population were studied with EPR. Such control groups have yielded background dose rates between 1 and 2 mGy per year for tooth enamel samples prepared from molars and premolars (*93*). Estimation of the background radiation component can be made by multiplication of the annual background dose rate and tooth age, which can be determined based on the tooth donor age and position of the tooth.

Another useful step in some studies is conversion of the dose absorbed in tooth enamel into estimates of organ doses. This requires complete knowledge of the dose distribution throughout the body. Typically this is solved through Monte Carlo calculations of radiation energy absorption by a mathematical model of the human body. Organs are usually represented by simplified models, or phantoms, which have density, shape and sizes close to actual organs. Unfortunately, there is no phantom for teeth, and calculations did not include the doses absorbed in tooth tissues for different geometries and types of exposure. It is only recently that several phantoms for different types of the teeth have been suggested (*103*), and Monte Carlo calculations have provided quantitative relationships between tooth enamel

dose and organ doses for external photon exposure of different energies.

Use of EPR in Epidemiological Studies

EPR dose reconstruction has been used to validate radiation exposure models, specifically to predict doses from radiation accidents or to determine environmental exposures. EPR dose reconstruction has been used for epidemiological studies of the atomic bomb survivors (*104*), Chernobyl accident (*101, 102*), Techa River population (*101*), Mayak nuclear workers (*94, 105*), and the Semipalatinsk population exposed as a result of nuclear tests (*106, 107*).

As mentioned previously, the reconstruction of doses obtained by both EPR and FISH for 100 survivors of the atomic bombs was closely correlated with radiation dose (*104*) and demonstrated the usefulness of EPR for acute exposures.

The lack of validated dose information has been one of the main problems in epidemiological studies of the consequences of the Chernobyl accident. Several thousand doses to individuals were reconstructed by EPR for different groups of Ukrainians and Russians residing in radioactively contaminated areas as well as for clean-up workers at the Chernobyl site. The EPR reconstructed doses to individuals exceeded doses estimated from background levels, e.g. up to 70 mGy for populations of some radioactively contaminated villages (*101*). EPR also revealed a mean whole-body dose of 160 mGy for clean-up workers (*102*).

The Techa River population was exposed as a result of radioactive waste releases into the river during the early 1950s. EPR dose reconstruction with teeth from Techa riverside residents revealed very high doses (up to 15 Gy) absorbed in tooth enamel for individuals born in 1945–1949 (*92*), whereas reconstructed doses for tooth donors born in other years were a factor of 50 lower. The former observation can be explained by the younger age of the donors born 1945–1949. Strontium-90 (^{90}Sr), which contributed about 12% of the isotopic compositions of radioactive releases into Techa River, is accumulated in teeth and bone. Therefore, individuals who had teeth formation during radioactive releases (1945–1949) accumulated a much higher amount of ^{90}Sr than other exposed individuals in that population. This finding suggests the ability of EPR dose reconstruction in teeth collected from donors of different ages to determine both the doses and type of radionuclide intake. The data from the subjects born in 1945–1949, who had accumulated ^{90}Sr, promises to provide important dosimetric information on exposure of the population to global radioactive fallout.

EPR dose reconstruction with teeth from Mayak nuclear workers showed relatively good agreement between EPR-derived doses and personal dose monitoring data (*94, 105*). The existence of reliable dosimetric information for Mayak nuclear workers made the results of the independent EPR dose reconstruction study valuable. It established an important bridge between doses measured by individual dosimeters and dose reconstruction results.

The Semipalatinsk population was exposed as a result of nuclear tests (456 nuclear explosions in the period between 1949 and 1989) (*106, 107*). EPR dose reconstruction is under way, and it probably will provide insights into the reliability of theoretical models for dose calculations.

Limitations

Some limitations of EPR dose reconstruction with tooth enamel include (1) the present need for tooth extraction; (2) the inability to distinguish radiation type, because both ionizing radiation (γ rays, β particles and X rays) and ultraviolet light produce essentially the same EPR signal; and (3) EPR is minimally sensitive to neutrons. The first disadvantage seems to be most serious limitation of the method. However, results from *in vivo* EPR have been reported recently (*108*). This approach does not require that the teeth be extracted, but it is less sensitive.

Future Directions

Currently, several potential applications of EPR retrospective dosimetry with teeth are in development. To overcome the necessity to have extracted teeth, an *in vivo* EPR retrospective dosimetry in L band (1.2 GHz) has been used (*108*). However, the lower limit of detection (LLD) is about 0.5 Gy (*108, 109*). Use of the Q band (37 GHz) for EPR dose reconstruction is a possible alternative, because it requires considerably smaller amounts of tooth enamel compared with X and L bands (*110*). This provides an important opportunity to use small portions ($\sim$10 mg) of tooth enamel, which can be harmlessly removed by a dentist without tooth extraction. The higher sensitivity of Q band should yield an improved LLD compared to X and L bands.

GLYCOPHORIN A SOMATIC MUTATION ASSAY (GPA)

Introduction

Ionizing radiation can induce mutations in human somatic cells, and accumulation of somatic cell mutations in humans has been linked to carcinogenesis. The GPA somatic mutation assay method was developed to provide a reliable and quick method to detect and measure somatic cell mutations in humans induced by exposure to ionizing radiation. Similar to the other two methods described in this paper, GPA was first applied to the A-bomb survivors and was found to be relatively well correlated with physically based dose estimates (*111–113*). Since that time, it has been applied principally to populations exposed to radiation from accidents (*18, 114–117*). Because the assay requires a small amount of blood and can be performed relatively quickly, it was thought that GPA would be suitable for identifying genotoxic exposures in large populations.

Fundamentals

Glycophorin A is a glycoprotein that is expressed on the cell surface of red blood cells; it occurs in two allelic forms, M and N. This somatic mutation assay uses a flow cytometric technique in which the glycophorin A protein is labeled with fluorescent monoclonal antibodies that are specific for individual allelic forms. The assay measures variant frequencies in the cell types (NØ, MØ, NN and MM) that express phenotypic loss of the glycophorin A allele, resulting from mutations in the glycophorin A gene in bone marrow progenitor cells. These variant frequencies serve as a quantitative indicator of radiation dose; the higher the frequency, the higher the cumulative radiation dose. Several comprehensive reviews of the GPA assay and its application to radiation-exposed populations are available (*83, 111, 118*).

Use of GPA in Epidemiological Studies

Past studies of the atomic bomb survivors and radiation accident victims have demonstrated a linear relationship between known dose and variant cell frequencies (GPA) after acute, high-dose, whole-body exposures to radiation (*111–113, 115*). Exposure to radiation from the Chernobyl accident was not associated with an increase in the GPA assay, after adjustment for smoking and age, for 625 Russian workers and 182 controls (*18*). The GPA assay was also applied to 734 Chernobyl clean-up workers and 51 controls from the Baltic countries to validate prior physical recorded doses for the workers (median dose 9.5 cGy). Again, no differences in variant frequencies of GPA between exposed and nonexposed clean-up workers were detected, most likely due to the low doses of radiation received by the workers (*116, 117*). To evaluate the utility of GPA as a biodosimeter of radiation doses accumulated over a long time, the GPA assay was applied to 36 radiation workers at the Sellafield Nuclear Facility who had received >50 mSv cumulative dose, based on previously recorded doses. No correlation was evident between variant frequency measured by GPA and radiation dose (*65, 119*). These results suggested that the GPA assay was not a reliable predictor of moderate or low-dose radiation exposure accumulated over a long period. A significant dose response of variant frequencies related to cumulative dose among hospital workers was noted in one study, but the results were likely influenced by a few persons with high cumulative doses (*120*).

Advantages

The GPA assay has several practical advantages. Only 1 ml of blood per subject is required. Blood collected from study subjects can be stored at refrigerator temperature (4°C) up to 1 week prior to analysis, making it useful in studies with limited resources available in the field conditions. The GPA assay can be performed on a commercially available flow cytometer, reducing the amount of labor and time, which makes it attractive for large population studies.

Limitations

A major limitation is that only 50% of the general population is M/N heterozygous and therefore eligible for the assay. No *in vitro* system for GPA exists by which to calibrate the assay. Interindividual variability, especially at doses above 1 Gy, make the GPA assay less useful as an individual biodosimeter than other techniques (*112, 113*). In a recent review of the usefulness of the GPA assay as a biological dosimeter of cumulative radiation exposure (*84*), the International Commission on Radiation Units and Measurements (ICRU) concluded that the GPA assay is not suitable for individual dose assessment, because of the interindividual variability of variant frequencies at similar doses, but the assay can be used to determine average doses in population groups.

Future Direction

Although the GPA assay has several practical advantages as a biological dosimeter, it does not appear to be useful as a biological dosimeter for external radiation doses less than 1 Gy. The assay may be useful in studies of populations exposed to higher radiation doses, especially when used in combination with other biological markers to characterize the level of radiation exposure.

CONCLUSION

Many epidemiological studies of the long-term risks of radiation exposure require large sample sizes to achieve adequate statistical power, and some of these studies involve study subjects in widely dispersed or remote geographic areas. To validate physical measurements, or to characterize the level of exposure in the absence of physical dosimetry, it is desirable for a biodosimeter to be reliable, simple and inexpensive. Although GPA probably best fits these criteria, it has proven to be most useful only at relatively high doses (≥ 1 Gy). Only 50% of a population would be eligible for this assay. Whereas chromosome painting (translocation analysis) can provide dosimetry estimates for lower doses on a population basis, it has not been possible to estimate doses with sufficient accuracy for individual subjects many years after exposure. Interindividual variability appears to be a limitation for both chromosome painting and GPA. EPR of tooth enamel is suitable for individual dose assessment at doses >30 mGy, but the present requirement for extracted teeth remains a disadvantage in many studies. Although the use of all three markers can contribute information on the level of exposure in irradiated populations exposed to external sources of radiation, an emphasis on the development of new biological markers using gene expression and biomarkers that can distinguish damage induced by high-LET radiation have been suggested by recent, expert reviews of radiation biodosimetry methods (*83, 121*).

APPENDIX 1

Conversion of Metaphases to Whole-Genome Cell Equivalents

For exchanges involving only two chromosomes, the fraction of chromosome exchanges observed is obtained by expanding the polynomial equation:

$$(p_1 + p_2 + p_3 + \ldots + p_n)^2, \quad \text{(A1)}$$

where the p_i's are the fractions of the genome painted in each unique color. In the simplest case involving two colors, expansion of the polynomial

$$(p_1 + p_2)^2 \quad \text{(A2)}$$

yields

$$p_1^2 + 2p_1p_2 + p_2^2. \quad \text{(A3)}$$

The fraction of all exchanges between two chromosomes is thus $2p_1p_2$ since this term in the equation represents exchanges between chromosomes labeled in colors p_1 and p_2. For three colors, the polynomial is

$$(p_1 + p_2 + p_3)^2 = p_1^2 + p_2^2 + p_3^2 + 2p_1p_2 + 2p_1p_3 + 2p_2p_3 \quad \text{(A4)}$$

and the fraction of all simple exchanges detected is

$$2p_1p_2 + 2p_1p_3 + 2p_2p_3. \quad \text{(A5)}$$

This proportion will always be less than one, meaning that cells analyzed by chromosome painting will miss the exchanges that occur between chromosomes labeled in the same color. To convert the number of chromosome exchanges observed by painting *N* cells to the number that (theoretically) would have been observed if every aberration had been detected, Eq. (A5) is multiplied by *N*,

$$N(2p_1p_2 + 2p_1p_3 + 2p_2p_3). \quad \text{(A6)}$$

For example, if 1000 metaphase cells are scored by painting and 56% of all exchanges are detected (see Fig. 2), then the data correspond to $1000 \times 0.56 = 560$ whole-genome equivalents. Similarly, if one desires to obtain chromosome painting data from 1000 whole-genome equivalents and 56% of all simple exchanges are detected, then $1000/0.56 = 1786$ painted metaphase cells must be scored. Conceptually, the more chromosomes that are painted and the more colors that are used, the greater will be the fraction of all exchanges detected. In a rigorous mathematical sense, the above equations hold true only for detecting simple exchanges, i.e. those between exactly two chromosomes. The probability of detecting exchanges involving more than two chromosomes will also increase with the number of chromosomes painted and the number of colors employed and will depend on the aberration complexity and type (e.g. simple break or exchange). For practical purposes, most conversions of metaphases scored to whole-genome equivalents use the formulas shown above, and differences in detection efficiencies for simple and complex rearrangements are ignored.

APPENDIX 2

Counting Clonal Cells

Mathematically, this is performed as follows:

$$F = (A + C)/N, \quad \text{(A7)}$$

where *F* is the frequency of translocations per cell, *A* is the number of cells with translocations (or insertions) that are not clonal, *C* is the number of translocation-bearing cells in the clone, and *N* is total number of cells scored for that subject. The adjusted frequency of translocations is

$$F_{adj} = (A + 1)/(N - C + 1). \quad \text{(A8)}$$

The difference in the results produced by Eqs. (A7) and (A8) may be negligible if the number of aberrations is large and the size of the clone(s) is small. However, this is not always the case (*39*, *57*, *58*). If the question being addressed concerns the mean dose to a population, one may adjust the individual data as described and then take the average.

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